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MOUSE MONOCLONAL ANTIBODIES FOR EXPERIMENTAL IMMUNOTHERAPY PROMOTES KILLING OF TUMOR CELLS

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Monoclonal antibodies (MAbs) produced against a human colon adenocarcinoma cell line, Colo-205, were tested in antibody-dependent macrophage-mediated cytotoxicity (ADMMC) assays. The antibodies C163, C215, C245 (IgG_{2a} isotype); C151, C239, C241, C242 (IgG_1); C152 (IgG_{2b}); and C50 (IgM) were evaluated for their ability to promote killing of Colo-205 cells by thioglycollate-elicited peritoneal mouse macrophages. The concentrations of antibodies tested in ADMMC assays ranged from 1.0 ng/ml to 100 μg/ml, and the concentration at which 50% of tumor cells were lysed was used to characterize each antibody. Antibodies of the IgG_{2a} isotype promoted the strongest macrophage-mediated tumor cell lysis effects *in vitro*. MAbs C215, C163, and C245 were also tested in nude mice which had been inoculated with Colo-205 cells. Tumor suppression was observed in mice injected with MAbs, supporting our ADMMC findings *in vitro*. Animals treated with MAbs had fewer and smaller tumors, and longer periods of latency between the inoculation of tumor cells and development of tumors, compared to mice sham-treated with PBS. However, in a study of established tumors, C215 antibody did not suppress tumor growth. Serum collected from MAb-treated mice promoted lysis of Colo-205 cells in ADMMC assays while serum from sham-treated mice did not.

Development of tumor-specific MAbs as reagents for diagnosis and treatment of human cancer has been the focus of much attention (Lindholm *et al.*, 1983*a*; Thompson *et al.*, 1983; Magnani *et al.*, 1982; Sears *et al.*, 1985). Some MAbs suppress development of syngeneic and xenogeneic tumors in normal and nude mice (Herlyn and Koprowski, 1982; Bernstein *et al.*, 1980; Seto *et al.*, 1983). In particular, antibodies of the IgG_{2a} isotype have been associated with strong anti-tumor proliferative effects. Macrophages are involved in many regulatory and defensive functions against cancer (Alexander, 1976; Den Otter *et al.*, 1983). Antibody-dependent suppression of tumor growth *in vivo* has, in a number of systems, been ascribed to macrophages as being effector cells responsible for tumor-cell killing. Herlyn and Koprowski (1982) indicated that the macrophage was the effector cell involved in suppression of tumor development in nude mice injected with MAbs.

We screened 9 murine MAbs representing 3 subclasses of IgG and IgM in ADMMC assays. The antibodies were developed against a human colon adenocarcinoma cell line, Colo-205. ADMMC assays are useful for studying and modelling the conditions by which tumor-cell lysis can occur. We show here that 3 IgG_{2a} isotype MAbs, C163, C215, and C245, in the presence of mouse peritoneal macrophage, PM, produced strong cytolytic effects against Colo-205 tumor cells in ADMMC assays *in vitro*, and suppressed tumor development in nude mice. Some of the conditions which affect tumor suppression were examined *in vivo* and *in vitro*.

MATERIAL AND METHODS

Colo-205 cell culture

Colo-205, a human colon adenocarcinoma cell line, was obtained from the American Type Culture Collection (ATCC), Rockville, MD, and maintained as a monolayer in standard medium. Standard medium was prepared in Iscove's modified Dulbecco's medium (Gibco Europe, Paisley, Scotland) supplemented with L-glutamine, 2 mM (Gibco); β-mercaptoethanol, 50 μM (Sigma, St. Louis, MO); L-asparagine, 36.0 mg/l; L-arginine-HCl, 116 mg/l; folic acid, 10 mg/l; sodium pyruvate, 110.1 mg/l (all from Gibco); fetal bovine serum, 10% (v/v) (Tissue Culture Services, Slough, UK), and buffered with HEPES and sodium bicarbonate, 3.024 g/l (Sigma). Cells were cultured in tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C, and incubated in a humidified atmosphere containing 5% CO_2 in air. Cells were removed from flasks using 0.1% trypsin (w/v) (Difco, Detroit, MI) in EDTA (Sigma), 0.02%. This procedure yielded cells which were ≥ 95% viable as determined by Trypan blue exclusion.

Animals

Thymus-deficient nude mice (*nu/nu* BALB/c or C57Bl/6 background), 5–8 weeks of age, were purchased from Bomholtgard, Ry, Denmark. Nude mice were housed under pathogen-free conditions in a laminar flow hood in sterilized cages with autoclaved food and water provided *ad libitum*. All animals used for each experiment were of the same sex and age. Normal BALB/c mice, 2–6 months of age, were also obtained from Bomholtgard.

Production and purification of MAbs

Hybridomas secreting MAbs were produced by fusion of Sp2/O myeloma cells and spleen cells obtained from Colo-205 immunized mice (Lindholm *et al.*, 1983*a*). Propagation and cloning were performed essentially as described by Fazekas de St. Groth and Scheidegger (1980). Briefly, MAbs were characterized for specificity first by selecting for reactivity against the immunizing cell line, and for non-reactivity with human peripheral blood lymphocytes. The method of Mancini *et al.* (1965) was used to determine isotype and concentration of antibody from isolated clones using single radial immunodiffusion in agarose (1.5% in NaCl), containing isotype-specific anti-mouse immunoglobulins (Lindholm *et al.*, 1983*b*). C163, C215, and C245 MAbs are of the IgG_{2a} isotype; C241, C242, C151, and C239 are IgG_1 isotype antibodies; C152 is an IgG_{2b} isotype antibody; and C50 is an IgM antibody. C50 has binding specificity for sialosyl lactotetraose and sialylated $Lewis^a$ antigen on the CA-50 glycoprotein expressed by Colo-205 cells. C241 also recognizes the same epitopes as does C50. Chemical characterization of the epitope defined by C239 has shown that it recognizes sialylated $Lewis^a$ antigen. C151 and C245 both recognize the $Lewis^a$ blood-group antigen. C215 and C163 antibodies recognize protein epitopes, and C242 and C152 MAbs identify different carbohydrate epitopes on Colo-205 cells. LM4, an IgG_{2a} isotype MAb described by Nilsson *et al.* (1985), does not bind to Colo-205 cells and was

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Abbreviations: ADMMC, antibody-dependent macrophage-mediated cytotoxicity; PM, peritoneal macrophage; PBS, phosphate-buffered saline; MAbs, monoclonal antibodies.

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included as a control in ADMMC assays. The antibodies used in this study were purified from ascites fluid after injecting hybridomas into normal BALB/c mice treated with pristane (2,6,10,14-tetramethylpentadecane, Aldrich, Milwaukee, WI). Ascites fluid was collected and precipitated with saturated ammonium sulfate to 40%. The precipitate was resolubilized in PBS (0.01 M sodium phosphate, 0.14 M NaCl, pH=7.2), dialyzed and purified by Protein A-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography (Lindmark *et al.*, 1983). MAbs were eluted off the column with an acid-glycine buffer (0.2 M glycine, 0.25 M NaCl; pH=3.5), neutralized with NaOH and dialyzed against PBS. Antibody purity was determined by agarose gel electrophoresis. Antibodies were stored at −20°C, and filter-sterilized before use.

$F(ab')_2$ fragments of C215 MAb were prepared by pepsin digestion following standard procedures (Lamoyi and Nisonoff, 1983), and purified by protein A-Sepharose column chromatography.

Induction of Colo-205 tumors in nude mice

Colo-205 tumor cells were prepared for injection into mice by trypsinization as described above. Cells were counted with a hemacytometer and the cell concentration was adjusted to 2×10^7 cells per ml in PBS. Tumor growth was induced in nude mice by s.c. injection of 2×10^6 cells in 0.1 ml PBS at 2 locations on the dorsal hindquarters. Mice were divided into groups of 5 to 13: generally there were 8–10 mice in each group.

Administration of MAbs to nude mice

Mice received daily injections of 200 μg MAb by i.p. injection immediately following the inoculation of tumor cells. As a control of sham-treated tumor growth, each experiment included a group of mice which were injected with PBS alone. To determine the effect of MAbs on tumor suppression in mice with established tumors, the first injection of MAb was delayed in some experiments up to 10 days following the inoculation of tumor cells. In these experiments the dosage of antibody was 400 μg per mouse per day, for 15 consecutive days.

Determination of tumor incidence, diameter, and weight

The incidence of tumors was recorded twice weekly and tumor diameter was measured using calipers along 2 perpendicular axes. The absence of tumor at a site of inoculation was similarly recorded. Mean tumor diameter and tumor incidence were determined over the entire experimental period. Mice were killed after a 30- to 40-day observation period after the last MAb injection, and tumors were excised and weighed. Tumor burdens were recorded as the total weight of tumor for each mouse, and the mean for each group of mice was determined.

Preparation of peritoneal macrophages

Peritoneal macrophages for ADMMC assays were elicited by i.p. injection of 1 ml Brewer's Thioglycollate medium, TG (Difco, Detroit, MI) into normal and nude mice, and collected by peritoneal lavage with 5 ml sterile sucrose (11.6% in PBS) solution 3–4 days later. Cells were washed twice in PBS and counted with a hemacytometer. Approximately 70–80% of the cells recovered were macrophages. The concentration of macrophages was adjusted to 1×10^6 per ml in standard medium, and 100 μl of the cell suspension were aliquoted into 96-well flat-bottomed plates (Falcon, Oxnard, CA). The plates were incubated for 1–2 hr at 37°C, and non-adherent cells were removed by washing each well with PBS. This procedure yielded a confluent monolayer of adherent cells, which were found to be 90–95% macrophages by non-specific esterase staining.

Labelling and preparation of Colo-205 cells for ADMMC assays

Colo-205 cells used for ADMMC assays were labelled with (6-^3H)thymidine (Amersham, Birkerød, Denmark), 2 μCi/ml, in standard medium. Label was added to flasks containing subconfluent monolayers of cells. After 24 hr, unincorporated label was removed by decanting the medium from the flask and washing the cells once in PBS. Colo-205 cells were trypsinized and counted. The cell concentration was adjusted to 4×10^5 cells per ml in PBS.

Tumor cells used for ADMMC assays were obtained after passage *in vitro* (as described above), or by dissociation of tumor tissue removed from mice treated with MAb. In the latter case, a single-cell suspension was prepared from freshly dissociated tumor tissue by pressing minced tissue through Nitex cloth and allowing the cell clumps to settle to the bottom of a conical test tube after about 5 min. The supernatant, containing mostly single cells, was collected and washed twice in PBS. The number of cells was adjusted to 1×10^5 per ml in standard medium. Cells were labelled as described above.

Antibody-dependent macrophage-mediated cytotoxicity assays

(6-^3H)thymidine-labelled Colo-205 tumor cells, 4×10^4 per 100 μl, were added in standard medium to each well of 96-well plates. The target-to-effector cell ratio in wells containing macrophages was 1:2.5. Dilutions of MAbs were prepared in standard medium at 10× concentration and diluted 1:10 in the assay wells. The final volume of medium per well was 250 μl, adjusted with standard medium. In all experiments, controls included (1) wells without antibody; (2) wells without macrophages; (3) wells with only tumor cells to measure spontaneous lysis of tumor cells. Maximum release of label by tumor cells was determined by solubilization of cells in sodium dodecyl sulfate, SDS, 2% (Bio-Rad, Richmond, CA).

For some ADMMC assays nude mouse serum, collected from both antibody-treated and sham-treated mice, was substituted for purified antibody. Approximately 8–12 hr following the last injection of MAb or PBS, serum was collected and heat-inactivated at 56°C for 30 min. Serial dilutions of mouse serum were made in standard medium containing 1% (v/v) normal mouse serum (Dakopatts, Copenhagen, Denmark) before addition to the test wells. The reactivity of MAb in mouse serum, and sensitivity of tumor cells to lysis, were tested in the presence of PM from both normal and nude mice.

After 48 hr, tumor-cell lysis was measured by collecting a 50-μl aliquot of supernatant fluid from each test well, diluting in scintillation cocktail (Insta-gel, Packard, Bandhagen, Sweden), and counting each sample in a β-counter. Tumor cell lysis was calculated as follows:

$$\text{percent cytotoxicity} = [(\text{experimental release of label}) - (\text{spontaneous release of label})]/[(\text{maximum release of label}) - (\text{spontaneous release of label})] \times 100.$$

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences in mean tumor weights and tumor diameters of mice treated with MAbs and control animals injected with PBS. Fisher's exact test was used to determine the significance of the differences in tumor incidence between treated and control animals. Probabilities of 5% ($p < 0.05$) or less were considered significant.

RESULTS

Effects of antibody isotype on ADMMC reactions in vitro

Monoclonal antibodies of different isotypes were tested in ADMMC assays *in vitro* for their ability to trigger tumor cell lysis by macrophages. The results of tumor cell lysis in the

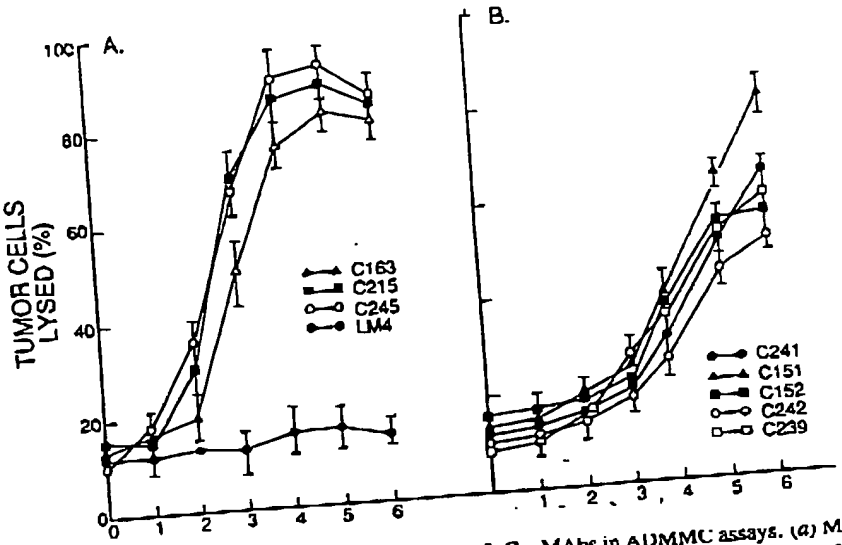

FIGURE 1 – Typical patterns of Colo-205 tumor-cell lysis for IgG_{2a}, IgG_1 and IgG_{2b} MAbs in ADMMC assays. (*a*) MAbs of the IgG_{2a} isotype, C215, C163 and C245. Lm4 MAb was included as a control since it does not bind to Colo-205 cells. Panel (*b*) compares antibodies C151, C241, C239, and C242 of the IgG_1 isotype, and C152 (IgG_{2b} isotype). MAbs were added to triplicate test wells at the following concentrations: (1) 0.001, (2) 0.01, (3) 0.1, (4) 1.0, (5) 10.0 and (6) 50 μg/ml with (6-^{3}H)thymidine-labelled Colo-205 tumor cells and PM. The amount of label released from tumor cells in the absence of antibody is shown on the Y-axis.

presence of MAbs are shown in Figure 1. Panel (*a*) illustrates the results of tumor-cell lysis triggered by the IgG_{2a} isotype MAbs tested: C163, C215, and C245. The percentage of tumor cells lysed was concentration-dependent and increased with increasing concentration of antibody in the presence of macrophages. Colo-205 tumor cells were not non-specifically sensitive to lysis by IgG_{2a} MAbs. LM4, an IgG_{2a} isotype antibody which does not bind to Colo-205 cells, did not trigger lysis by macrophages. Panel (*b*) illustrates results of ADMMC assays using C152, C151, C241, C239, and C242 MAbs. These MAbs, of the IgG_{2b} and IgG_1 isotypes, were associated with lower levels of tumor-cell lysis compared to IgG_{2a} isotype antibodies at the same concentration. Tumor-cell lysis in the presence of an IgM MAb, C50, and of PM was not significantly above background or spontaneous levels observed in the absence of antibody (data not shown). None of the MAbs tested was observed to lyse tumor cells directly, nor was lysis by macrophages, independent of antibody, observed.

The effectiveness of different MAbs of same and different isotypes was compared in ADMMC assays. Antibody-dependent lysis of tumor cells was characterized by the concentration at which 50% of tumor cells were lysed. The results are shown in Table I. In general, MAbs of the IgG_{2a} isotype were more effective in ADMMC assays than the other isotypes of antibodies tested, as evidenced by the lower concentration of antibody required to produce 50% tumor-cell lysis. This suggested that IgG_{2a} isotype antibodies would be interesting for further study of tumor suppression in nude mice.

TABLE I – CHARACTERIZATION OF MAbs OF IgG_1, IgG_{2a} AND IgG_{2b} ISOTYPES BY THE CONCENTRATIONS AT WHICH 50% OF COLO-205 TUMOR CELLS WERE LYSED IN ADMMC ASSAYS

Antibody	Isotype	C_{50} (μg/ml)
C163	IgG_{2a}	0.1–1.0
C215	IgG_{2a}	0.01–0.1
C245	IgG_{2a}	0.01–0.1
C152	IgG_{2b}	10–50
C239	IgG_1	10–50
C241	IgG_1	10–50
C242	IgG_1	1.0–10
C151	IgG_1	10–50

The concentration at which 50% of Colo-205 tumor cells were lysed in the presence of PM from normal BALB/c mice was used to characterize each antibody's effectiveness in ADMMC assays. These values were taken from assays using ^{3}H-thymidine-labelled Colo-205 tumor cells after 48 hr incubation.

Effect of MAbs on tumor growth in nude mice

MAbs C163, C215, and C245 were tested, and compared to sham-treated mice, for their ability to suppress tumor growth in nude mice. The effects were measured in mice injected with antibody or PBS immediately following inoculation of Colo-205 tumor cells. Each mouse received injections of 200 μg for 9 or 18 consecutive days. The mean tumor diameters in mice treated with MAbs for 9 days were significantly lower than those in sham-treated mice (Fig. 2). All mice in the PBS group had to be killed on day 23 because of their large tumor burden. However, mice in the groups which had received either of the 3 MAbs were still quite healthy, even though small tumors had developed on some of the animals. On day 55, mice in the 3

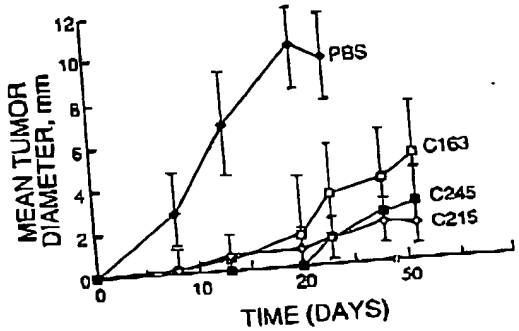

FIGURE 2 – Comparison of mean tumor diameter in nude mice receiving daily injections of C215, C163, and C245 MAbs, or PBS. After inoculation of tumor cells, at time 0 (To), mice were injected with PBS, or MAb, for 9 consecutive days. A significant difference in mean tumor diameter was determined (on day 23) upon comparing mice treated with MAb to mice sham-treated with PBS ($p \leq 0.001$, Student's *t*-test). There were no significant differences comparing mean tumor diameters of mice injected with C215, C163, or C245 MAbs.

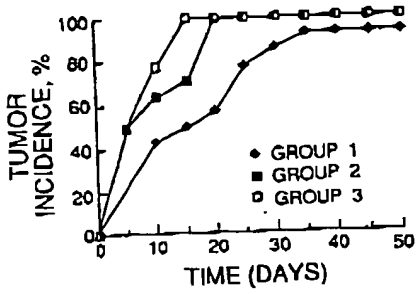

FIGURE 3 – Effects of delaying administration of C215 MAb for 5 and 10 days following inoculation of Colo-205 tumor cells in nude mice. Mice were inoculated with Colo-205 cells and divided into 3 groups. Mice in groups 1 and 2 received the first injection of C215 5 and 10 days, respectively, after the inoculation of tumor cells. Mice in group 3 were injected with PBS.

antibody-treated groups were killed and the tumors removed and weighed. Tumor weights from mice injected with MAbs were compared to those of mice injected with PBS and significant differences were observed. The mean total weight of tumors removed from animals injected with MAbs was 0.3 ± 0.2 g, compared to 1.03 ± 0.2 g from mice injected with PBS ($p=0.001$, Student's t-test). In addition, mice treated with MAb had an approximately 3-fold lower tumor incidence than sham-treated mice. Tumor incidence in MAb-treated mice ranged between 0–35% on day 55, while in PBS-injected mice, tumor incidence was 100% by day 8. Overall, mice injected with antibodies had smaller mean tumor diameters, smaller mean tumor weights, lower tumor incidence, and a longer survival time compared to PBS, control animals.

Effect of C215 MAb on established tumors

To determine whether C215 would be effective against established, palpable tumors, the first injection of MAb was delayed up to 10 days following the inoculation of tumor cells in nude mice. In a previous experiment, tumors were visible in sham-treated mice by day 8. Delaying the onset of antibody therapy was planned to coincide with the period just before and just after tumors became visible. The dosage of C215 MAb was increased to 400 μg daily. The results of using C215 MAb against established tumors are shown in Figure 3. Mice which received their first antibody injection on day 5 (group 1) had a slightly lower incidence of tumor than mice injected with antibody 10 days after inoculation of tumor cells (group 2), but this difference was not significant. Furthermore, comparison of mean tumor weights at the termination of the experiment did not produce significant differences between the treated and sham-treated groups (data not shown).

Tumor-cell sensitivity to lysis in ADMMC assays with serum collected from treated mice

Tumors which developed in mice treated with C215 MAb immediately after tumor cell inoculation were examined for sensitivity to lysis in ADMMC assays. As shown in Figure 4, tumor-cell lysis was demonstrated in the presence of serum collected from mice which had received daily injections of 200 μg C215 MAb for 18 consecutive days. PM were harvested from nude mice which had been treated with C215 antibody. Controls included serum collected from sham-treated mice injected with PBS and purified C215 antibody. Freshly dissociated Colo-205 tumor cells were lysed in a dose-dependent manner in serum from mice injected with C215, or with purified C215 MAb, but not in serum collected from sham-treated mice. The data imply that tumor growth in treated mice was not the result of loss of antigen sensitivity to antibody. Tumor-cell lysis by heat-inactivated serum or purified antibody in the absence of PM was not observed.

The effect of C215 F(ab')$_2$ fragments in ADMMC assays with intact C215 antibody

F(ab')$_2$ fragments of C215 MAb were used in ADMMC assays to block tumor-cell lysis in the presence of intact C215 antibody. The results are shown in Figure 5. C215 F(ab')$_2$ fragments alone did not promote tumor-cell lysis in ADMMC assays, indicating that the Fc portion of the molecule was critical in ADMMC reactions. C215 F(ab')$_2$ fragments inhibited Colo-205 cells from being lysed in the presence of intact C215 antibody only when C215 F(ab')$_2$ fragments were preincubated with tumor cells before the addition of intact C215 antibody. When intact C215 antibody and F(ab')$_2$ fragments were added into the test wells simultaneously, or at the same concentrations (10 μg/ml), the inhibitory effect was no longer observed: Colo-205 cells were lysed under these conditions.

DISCUSSION

We compared 9 murine MAbs for their ability to promote lysis of Colo-205 tumor cells in ADMMC assays, and observed that the highest percentage of tumor-cell lysis varied with antibody isotype in the presence of PM. Maximum (100%) levels of tumor-cell lysis were observed only in the presence

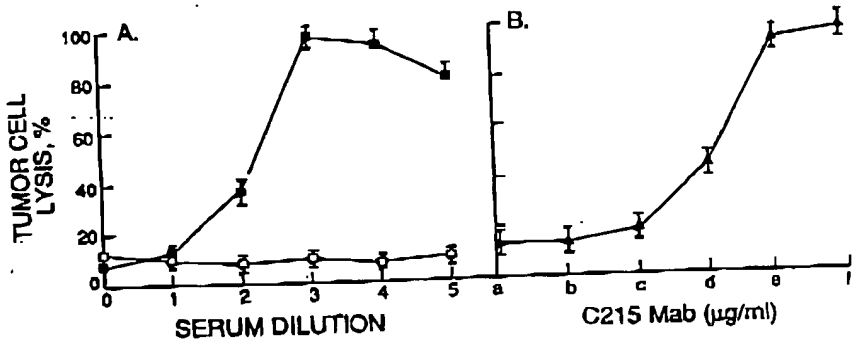

FIGURE 4 – Determination of susceptibility of Colo-205 tumor cells to cytolysis in ADMMC. Tumors which developed in mice treated with C215 MAb were dissociated and the tumor cells incubated with PM from nude mice. In panel (*a*), lysis was compared in the presence of serum collected from mice injected with C215 (200 μg per mouse per day) for 18 consecutive days (■), or of serum collected from mice injected with PBS (□). In panel (*b*), lysis in the presence of purified C215 MAb is shown. Serum samples were tested at the following dilutions; (1) 1:100,000, (2) 1:10,000, (3) 1:1,000, (4) 1:100, and (5) 1:10. Purified C215 antibody was present in the following concentrations; (a) 0, (b) 0.001, (c) 0.01, (d) 0.1, (e) 1.0, and (f) 10 μg/ml. Tumor-cell lysis was determined as described in "Material and Methods".

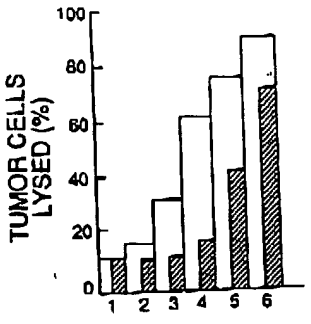

FIGURE 5 – Colo-205 cells were pre-incubated with F(ab')$_2$ fragments of C215 antibody (10 μg/ml) for 1–2 hr at 4 °C and added to triplicate wells containing PM and intact C215 antibody in the following concentrations: (1) 0, (2) 0.001, (3) 0.01, (4) 0.1, (5) 1.0, and (6) 10.0 μg/ml. Lysis of Colo-205 cells in the presence of intact antibody was compared under conditions in which cells were pre-treated with F(ab')$_2$ fragments (▨), and cells not pre-treated with F(ab')$_2$ fragments (□).

of IgG_{2a} isotype MAbs tested. MAbs of the IgG_1 or IgG_{2b} isotypes were less efficient in ADMMC assays than antibodies of the IgG_{2a} isotype at the same concentration. Using the concentration at which 50% of all tumor cells were lysed as the basis of comparison to characterize the effectiveness of different MAbs in ADMMC assays, we observed a significantly higher effectiveness of IgG_{2a} isotype antibodies. High levels of tumor-cell lysis in the presence of IgG_{2a} isotype antibodies occurred at concentrations 10–1,000 times lower than those of IgG_1 or IgG_{2b} isotype MAbs. Strong tumor-suppressive effects were also observed in nude mice injected with MAbs of the IgG_{2a} isotype. Tumor incidences in antibody-treated mice were significantly below those seen in control animals. This observation was predicted by ADMMC experiments *in vitro*, and supports work by other investigators (Herlyn and Koprowski, 1982; Adams *et al.*, 1984).

It is not known why MAbs of the IgG_{2a} isotype are generally more potent than other isotypes as mediators of tumor-cell lysis in ADMMC assays and inhibitors of tumor development in animal models. A number of hypotheses have been proposed. There are distinct Fc receptors for different subclasses of IgG on the surface of macrophages (Diamond and Yelton, 1981; Unkeless *et al.*, 1981). Perhaps the unique structure of the IgG_{2a} isotype antibody triggers macrophage responses for killing tumor cells in ways which are functionally distinct from those promoted by antibodies of different isotypes. Selective enhancement of the Fc receptor (FcRI) for IgG_{2a} isotype antibodies has been reported in macrophages activated for tumor-cell killing (Ezekowitz *et al.*, 1983). Johnson *et al.* (1986) observed that trypsinization of macrophage results in decreased lysis in ADMMC assays. A structure-function relationship may exist between the expression of a particular receptor on macrophages and their ability to kill tumor cells. Many questions remain concerning the functional association of different Fc receptors for IgG, and to what extent receptors can co-modulate. Steplewski *et al.* (1985) have shown that class-switch variants of 19-9 MAb differ in their ability to mediate ADMMC-reactions *in vitro*, and suppress tumor development in nude mice; IgG_{2a} isotype variants were superior, however. This work has shown that changing the Fc portion of an antibody while retaining binding specificity and affinity for epitopes changed the effectiveness of that antibody to mediate tumor-cell killing. Further studies on the killing mechanism(s) activated in macrophages upon binding different classes of antibody are necessary.

The observation that ADMMC reactions were concentration-dependent suggested that a "threshold effect" existed concerning the amount of MAb which must be bound to a tumor cell before lysis is observed. IgG_{2a} isotype antibodies vary in their ability to promote ADMMC, target lysis occurring only when some minimum, or critical, density of antibody had bound to the tumor cell surface (Herlyn *et al.*, 1985). The fact that all 3 IgG_{2a} isotype antibodies used in our study were associated with strong tumor-suppressive effects was probably due to the antigens recognized by these antibodies being highly expressed.

Using F(ab')$_2$ fragments prepared by pepsin digestion of C215 MAb, we found that the ADMMC response was abrogated. Presumably, the negative result was due to the lack of the Fc portion of the Ig molecule. F(ab')$_2$ fragments were demonstrated in ELISA to retain binding specificity for the target cells (data not shown). ADMMC experiments using F(ab')$_2$ fragments were designed to block, or reduce, tumor-cell lysis in the presence of intact C215 MAb. However, this effect only occurred after pre-treatment of the target Colo-205 cells with F(ab')$_2$ fragments. F(ab')$_2$ fragments were not found to competitively inhibit tumor-cell lysis in the presence of intact antibody. The inhibitory effect by F(ab')$_2$ fragments is believed to have occurred as the result of a critical number of binding sites on the tumor-cell surface being occupied before addition of intact antibody.

The lack of effect of MAbs against established tumors indicated that timing was critical. Immunotherapy was most effective against non-established tumor cells, when administration was begun just after inoculation of tumor cells. Allowing tumor cells to establish and proliferate before initiating antibody administration resulted in tumor incidences which were not significantly different from those of sham-treated mice. Whether activated host effector cells were physically deficient in established, growing tumors is not known. A limitation in the management of growing tumors is the number and functional status of effector cells *in situ* (Shin *et al.*, 1976; Matthews *et al.*, 1985). Induction of regression of established tumors seems to be limited by the size of the tumor. The reasons for this could be that factors secreted by large tumors inhibit macrophages from entering, or functioning within, the tumor, or that the amount of antibody, or the number of activated effectors, are limited in relation to the number of tumor cells. A mouse with a large tumor can suppress smaller tumors induced at different sites, indicating that effector shortages within large tumors are not due to systemic exhaustion and that antibody is not the limiting factor (Shin *et al.*, 1978). The significance of intra-tumoral cytotoxic macrophages has been reviewed by Den Otter *et al.* (1983).

Stability, clearance, and degradation of mouse MAbs are factors which can influence therapeutic effectiveness against tumors. Production of allogeneic or xenogeneic antibodies would limit their potential effectiveness in the host. How much MAb actually reaches the tumor site through the circulation, or is necessary for suppression of tumor development, however, is not known. The concentration of antibody in the tumor depends upon antibody transport through the circulation and diffusion, and may not be accurately represented by examining circulating levels. Nevertheless, development of tumors in treated mice was examined to determine if (1) free MAb was present in mouse serum, and (2) antigen recognized by injected antibody was present on the tumor cell surface, or if the cell had become antigen-negative through modulation or shedding of antigen. The antigen recognized by C215 antibody was found to be stable on *ex vivo* tumor cells from mice which had been treated with C215. Tumor-cell lysis was demonstrated in ADMMC assays in the presence of serum collected from nude mice which had been injected with C215, but not with serum collected from sham-treated mice. Immunohistochemical analysis of tumor tissue taken from the same tumors dissociated

for ADMMC assays demonstrated strong and uniform staining with biotin-labelled C215 MAbs (data not shown). Therefore, we found no indication that development of tumors in treated mice was due to weak or absent expression of antigen on the tumor cell surface, or that serum antibody levels were insufficient. However, we do not know whether or not nude mice injected with allotypic antibodies mount an anti-antibody response.

Immunological approaches using MAbs to assist in the treatment or management of cancer would appear to be most successful when detected early, or when used to enhance other therapeutic techniques intended to reduce the tumor burden. The data presented above indicate that passively administered MAbs would be ineffective in treating advanced cancer. MAbs may be better suited as adjuvant therapeutic agents: following surgical removal of the primary tumor, or in combination with immunomodulators which potentiate the host's immune response. Thorough understanding of the conditions which lead to tumor-cell lysis *in vitro* might enable researchers to predict the potential outcome of an interaction between antibody, effector cell, and tumor cell *in vivo*, and hence lead to the design of more effective treatment protocols. Furthermore, combinations of MAbs which exploit different effector mechanisms, cellular and complement-mediated, may prove to be more effective than administration of individual MAbs.

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